Trehalose Liposomes Inhibit the Growth of Glioblastoma Cell In vitro and In vivo

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ABSTRACT

The inhibitory effects of Trehalose Liposomes (TL) composed of α-D-glycopyranosyl-α-D-glucopyranoside monomyristate and L-α-dimyristoylphosphatidylcholine on the growth of human glioblastoma (U-87MG) cells were evaluated. Induction of apoptosis was observed after fusion and accumulation of TL in U-87MG cell membranes. Increased membrane fluidity of U-87MG cells treated with TL was observed. TL caused apoptosis in U-87MG cells through the activation of mitochondria and apoptosis-inducing factor via a caspase-independent pathway. Tumor weights markedly decreased in orthotopic graft mouse models of glioblastoma (U-87MG) after intravenous administration of TL compared with those in the control group.

KEYWORDS: Trehalose liposome; Nanomedicine; Therapeutic effects; Glioblastoma

ABBREVIATIONS

DMPC: L-α-dimyristoylphosphatidylcholine; TL: Trehalose Liposomes composed of DMPC and TreC14; TreC14: α-D-glycopyranosyl-α-D-glucopyranoside monomyristate

INTRODUCTION

Glioblastoma is the most malignant brain tumor. The standard of care is a combination of radiotherapy and chemotherapy with temozolomide after surgical resection [1]. Treatment with bevacizumab during radiotherapy does not improve survival in patients with glioblastoma [2, 3]. Trehalose Liposomes (TL) composed of L-α-Dimyristoylphosphatidylcholine (DMPC) and trehalose micelles have been produced using a sonication method [4, 5]. TL are effective in inhibiting the growth of tumor cells without including antitumor agents In vitro and In vivo [6-8]. Induction of apoptosis has been observed in colon and gastric cancer, hepatocarcinoma, leukemia, lung carcinoma, and breast cancer after treatment with TL [4-8]. The anti-invasive effects of TL on the proliferation of lung carcinomas have been reported [9]. However, the inhibitory effects of TL on the growth of glioblastoma cells have not yet been examined. In this study, we investigated the inhibitory effects of TL on the growth of glioblastoma (U-87MG) cells In vitro and In vivo.
Assessment of half-maximal inhibitory concentration (IC50) of TL in vitro

The IC50 of TL for U-87MG cells was determined using a WST-8 (2-[4-(2-methoxy-4-nitrophenyl)phenoxy]-2H-5(2H)-tetrazolium, monosodium salt) assay (Cell Counting Kit-8, Dojindo Laboratories, Kumamoto, Japan) [10]. U-87MG cells (~5.0 × 10^3 cells/ml) were seeded in 96-well plates and cultured in a 5% CO2 humidified incubator at 37°C for 24 h. Cells were cultured for a further 48 h after the addition of DMPC (0.20 × 10-3 M) and TL (0.60 × 10-4 M). The WST-8 solution was then added and incubated for 3 h. The absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific). The inhibitory activity of TL on the growth of U-87MG cells was evaluated by calculating Amean/Acontrol, where Amean and Acontrol denote the absorbance of water-soluble formazan in the presence and absence of TL, respectively.

Analysis of induction of apoptosis by TL

Cells were seeded at a density of 5.0 × 103 cells/100 ml in a culture dish and incubated in a humidified atmosphere of 5% CO2 at 37°C for 24 h. TL (0.1 × 10-4 M) was added to each culture dish and incubated in a humidified atmosphere of 5% CO2 at 37°C for 24 h. The cells were then treated with TL/NBDPC ([DMTre]=9.6 × 10-5 M, [NBDPC]=3.9 × 10-6 M~4.0 × 10-6 M) for 1-60 min and examined by flow cytometry using a 15 mW 488 nm air-cooled Ar laser and FL1 sensor (505-545 nm).

Measurement of cellular membrane fluidity

The membrane fluidity of U-87MG cells was measured by spectrophotometry (F-7100; HITACHI, Tokyo, Japan) based on a previously described fluorescence depolarization method [12]. The cells were labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH; Molecular Probes, Eugene, OR, USA) in a dark room. The fluorescence depolarization (P) value of DPH in cells treated with TL (5.0 × 10-5 M) was measured.

Assessment of apoptotic cells using flow cytometry

U-87MG cells (5.0 × 10^4 cells/ml) were treated with TL. After 24 h of incubation, each substrate solution (PhiPhiLux-GID2, CaspaseLux 8-LID2, CaspaseLux 9-MID2 for caspase-3, caspase-8, and caspase-9, respectively; OncolImmunin Inc., Gaithersburg, MD, USA) was added to the U-87MG cell suspension [11]. The cells were incubated for 45 min at 37°C, washed twice with 1 ml of ice-cold PBS (-), and resuspended in 1 ml fresh PBS (-). Caspase activity was analyzed by flow cytometry. Stained U-87MG cells were examined by flow cytometry using a 15 mW 488 nm air-cooled Argon laser and an FL1 sensor (505-545 nm).

Mitochondrial membrane potential

3, 3-Dihexyloxacarbocyanine iodide (DiOC6 (3); Molecular Probes, Eugene, OR, USA) was added to U-87MG cells (5.0 × 10^4 cells/ml), previously treated with TL (2.5 × 10-4 M), to evaluate mitochondrial transmembrane potential (Δψm). Stained U-87MG cells were examined by flow cytometry using a 15 mW 488 nm air-cooled Ar laser and an FL1 sensor (505-545 nm).
Statistical analysis

Data are presented as mean ± S.D. For statistical comparison, Student’s t-test was performed. Differences were considered statistically significant at P<0.05.

RESULTS

Physical properties of TL

The diameter of the TL was measured using dynamic light scattering measurements. The hydrodynamic diameter of TL was <100 nm, indicating that TL can avoid recognition by the reticular endothelial system in vivo [14]. The DMPC liposomes were unstable and precipitated.

TL inhibition of U-87MG cell growth leading to apoptosis

The 50% Inhibitory Concentration (IC50) of TL is shown in Figure 1A. The IC50 value was 92 μM, whereas that of the DMPC liposomes was 240 μM. The difference was significant, indicating a greater inhibitory effect of TL compared with that of DMPC liposomes. Induction of apoptosis was investigated using flow cytometry. The amount of apoptotic DNA increased in U-87MG cells treated with TL and reached 100% (Figure 1B).

Apoptotic signal transduction by TL

The apoptotic pathway in U-87MG cells treated with TL was examined. Caspase-3, -8, and -9 were not activated in U-87MG cells treated with TL, indicating that TL induced apoptosis through a caspase-independent pathway (Figure 2A). The mitochondrial pathway in U-87MG cells treated with TL was also examined. The mitochondrial transmembrane potential of U-87MG cells treated with HL decreased, indicating that TL induced apoptosis in U-87MG cells through the mitochondria (Figure 2B). AIF is involved in initiating the caspase-independent pathway of apoptosis. Thus, the expression of AIF was assessed. The fluorescence intensity of AIF increased in U-87MG cells treated with TL, indicating that AIF is involved in apoptosis induced by TL (Figure 2C). These results suggest that TL induces apoptosis through mitochondria and AIF in a caspase-independent pathway manner.

Accumulation of TL in cell membrane

The fusion and accumulation of TL membranes of U-87MG cells was examined using fluorescence probe-labeled TL. The fluorescence intensity of labeled TL increased (Figure 3A), indicating that TL fused and accumulated in the membrane of U-87MG cells.
The membrane fluidity of the U-87MG cells treated with TL was examined. The *P* value decreased for U-87MG cells treated with TL, indicating that the membrane fluidity of U-87MG cells increased after TL treatment (Figure 3B).

**DISCUSSION**

The findings are the first evidence of the therapeutic effect of TL in an orthotopic graft mouse model involving U-87MG human glioblastoma cells. There are five noteworthy findings. First, TL with a hydrodynamic diameter <100 nm persisted for 4 weeks. Second, TL inhibited the growth of U-87MG cells. This inhibition was driven by increased apoptosis. Third, TL induced apoptosis in U-87MG cells via the release of AIF in a caspase-independent pathway manner. Fourth, TL fused and accumulated in U-87MG cell membranes, increasing their fluidity. Fifth, TL also inhibited tumor enlargement in an orthotopic graft mouse model of glioblastoma.

**CONCLUSION**

The findings are the first evidence of the therapeutic effect of TL in an orthotopic graft mouse model involving U-87MG human glioblastoma cells. The results could be potentially advantageous for future clinical applications of TL therapy in patients with glioblastoma.

**CONFLICT OF INTEREST**

No potential conflict of interest was reported by the authors.

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